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NEW MEDIUM FOR THE CULTURE OF RABBIT SPERM FOR TOXICOLOGY TESTING

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RESEARCH AND TECHNOLOGY DIRECTORATE

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A simple salts medium with only glucose as the energy source and buffered by Tris.HCl or HEPES has been developed for the culture of rabbit sperm cells. Cells remain motile for 20 hr and develop hyperactivated motility after incubation at 37 °C for 0.5-1 hr in air. The motility characteristics and parameters such as Vc, AALH, and linearity of the hyperactivated and nonhyperactivated cells are not different from those of cells cultured in the commonly used CO_2-carbonate buffered media such as DM and T6. This minimal medium extends the use of the rabbit sperm cell for in vitro toxicity testing to include volatile compounds and compounds that form insoluble carbonates, sulfates, or phosphates. Also, the system can be used to assess the potential antifertility effects of chemicals by measurement of changes induced in development of hyperactivated motility by the chemicals.

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PREFACE

The work described in this report was authorized under Project No. 1N6A. This work was started in November 1990 and completed in August 1992.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," National Institute of Health Publication No. 85-23, 1985, as promulgated by the committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council (Washington, DC). These investigations were also performed in accordance with the requirements of AR 70-18, "Laboratory Animals, Procurement, Transportation, Use, Care, and Public Affairs," and the Laboratory Animal Use and Review Committee (LAURC), U.S. Army Chemical Research, Development and Engineering Center (CRDEC),* which oversees the use of laboratory animals by reviewing for approval all CRDEC research protocols requiring laboratory animals.

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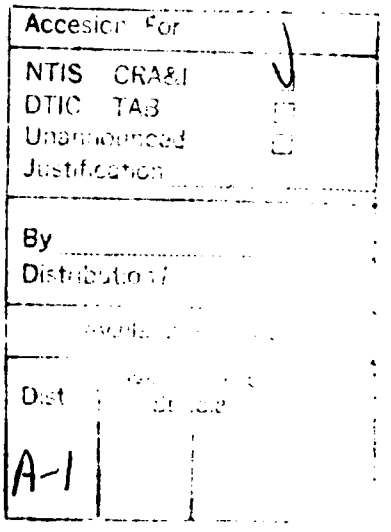

*CRDEC is now known as the U.S. Army Edgewood Research, Development and Engineering Center.

QUALITY ASSURANCE

This study, governed by protocol number 210910430000, was examined for compliance with Good Laboratory Practices as published by the U. S. Environmental Protection Agency in 40 CFR Part 792 (effective 18 September 1989). The dates of all inspections and the dates the results of those inspections were reported to the Study Director and management were as follows:

Phase Inspected	Date Inspected	Date Reported to Study Director/Manangement
Video-taping	05 Nov 1991	05 Nov 1991
Final Report	28 Jun 1993	30 Jun 1993

To the best of my knowledge, the methods described in this report were the methods followed during the study as indicated by the raw data found in the laboratory notebook. The report was determined to be an accurate reflection of the raw data recorded.

Kenneth P Cameron

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Quality Specialist
Life Sciences Department

30 June 1993

Date

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NEW MEDIUM FOR THE CULTURE OF RABBIT SPERM FOR TOXICOLOGY TESTING

1. INTRODUCTION

Rabbit sperm cells are usually cultured in the defined medium (DM) of Brackett and Oliphant.[1] Recent work has demonstrated that medium T6[2,3] is superior to DM in prolonging sperm cell viability and in developing hyperactivated motility.[4] The T6 medium, in common with others routinely used for cell culture, is CO_2-carbonate buffered. These media also contain phosphate and sulfate ions and frequently an organic acid as nutrients. Such media may present difficulties when used for the culture of cells in in vitro toxicology testing. For example, the compound under test may be volatile or form a precipitate with the anions. To overcome these impediments, a simple nonbicarbonate-carbonate buffered defined salts medium, which would support rabbit sperm cell motility, was sought. These studies are described in this report. In this report, hyperactivated motility refers to the motion patterns of the sperm cells without any implications as to the biological status of the cells.

2. MATERIALS AND METHODS

2.1 Animals.

New Zealand white rabbits were individually housed in standard rabbit cages in a room maintained at 25 ± 3 °C and 50 ± 10% relative humidity (RH) with a 12 hr light/dark cycle. Standard certified laboratory rabbit chow and water were available ad libitum.

2.2 Collection and Purification of Sperm Cells.

Sperm cells were collected and purified by centrifugation through a discontinuous Percoll gradient as previously described.[5]

2.3 Videotaping of Sperm Cells.

Videotaping of sperm motion was carried out as previously described.[6,7,8] A chamber, 20 μm depth, or Microcell slides, 20-24 μm depth, was used for videotaping.

2.4 Analysis of Videotapes.

Analysis of videotapes for hyperactivated sperm cells with the CellSoft system was carried out as described previously.[4] The following settings were used for the Cell Track

system with the VP110 video processor, CellTrak software version 3.15 and CTS/R version 3.21 (Motion Analysis System, Santa Rosa, CA).

- Frame rate 30 frames/s
- Duration of capture 30 frames
- Minimum path length 15 frames
- Minimum velocity 20 μm/s
- Maximum velocity 500 μm/s
- Distance scale factor 1.8393 μm/pixel
- Camera aspect ratio 1.0
- ALH path smoothing factor 7 frames
- Centroid X search neighborhood 4 pixels
- Centroid Y search neighborhood 2 pixels
- Centroid cell size 2 pixels minimum 25 pixels maximum
- Maximum path interpolation 1 frame
- Path prediction percentage 0%
- Sample depth 20 μm

2.5 Medium.

The compositions of media that were used are as follows.

- Saline (S) 0.9% NaCl, glucose 2.5 mg/mL
- Saline-Tris (ST) 0.9% NaCl-0.05M Tris-HCl, pH 7.4, glucose 2.5 mg/mL
- Medium M KCl 0.03 g, $CaCl_2.2H_2O$ 0.033 g, $MgCl_2.6H_2O$, 0.0106 g, NaCl 0.759 g, Tris.HCl 0.0572 g, Tris 0.0166 g, glucose 0.25 g, and bovine serum albumin (BSA) 300 mg in 100 mL twice glass distilled water
- Medium P same as M except that Tris and Tris-HCl are omitted, and NaCl is 0.789 g
- Medium H same as M except that Tris and Tris-HCl are replaced by HEPES (0.119 g) and the pH adjusted to 7.4 with NaOH

2.6 Sperm Cell Incubation.

Sperm cells purified by centrifugation through a Percoll gradient were washed with the medium to be used for incubation (5 min, 300xg, room temperature) and then resuspended in the medium at 5-13x10^6 cells/mL. Cells were also purified by two washes in the incubation medium. For experiments comparing response to medium, the ejaculate was divided into several parts and each part washed with the appropriate medium before resuspension in the medium for study. Cells resuspended in T6 were incubated at 37 °C in 95% air, 5% CO_2; otherwise, incubation was carried out at 37 °C in air. At time intervals after commencement of incubation, 5-7 μL of the cell suspension was placed in a 20 μm deep chamber or a 20-24 μm deep MicroCell (10 μL) and cell motility was observed under negative phase contrast at 100x.

2.7 Chemicals.

Bovine serum albumin, fatty acid free, Tris, Tris.HCl, HEPES, polyvinyl alcohol and polyvinlyprrolidone, and MW 10,000 were obtained from Sigma Chemicals Company (St. Louis, MO).

3. RESULTS

3.1 Medium Composition.

Rabbit sperm cells were not viable in saline (Table 1) or in saline buffered with Tris.HCl. Survival was improved in minimal medium (P) or minimal medium buffered with Tris.HCl (M), except for sperm cells from one or two rabbits in the colony (Table 1). When a macromolecule was present, motility and viability was good to excellent and sperm from all rabbits were viable for up to 20 hr (Table 1). Motility and viability were best in the presence of BSA.

3.2 Hyperactivated Motility.

Hyperactivated motility developed within 1 hr after commencement of sperm cell incubation in P or M medium. There was some variation among rabbits in the time after commencement of incubation for the appearance of hyperactivated motility, the percentage of motile sperm cells possessing hyperactivated motility, and the duration of hyperactivated motion. In general, hyperactivated motility appeared after 0.5 hr incubation, reaching a maximum in 1 hr before returning to progressive and circular motility after 2-4 hr incubation. Sperm cells from several rabbits did not develop hyperactivated motility, while those from a small number (e.g., rabbit 038) were virtually all hyperactivated and/or remained hyperactivated for longer than 6 hr. As expected from previous studies,[4,7,8] sperm cells, except

those from rabbit 038 and incubated in Medium T6, developed hyperactivated motility in significant numbers only after 6 hr incubation. The experiments were repeated several times for each rabbit and the behavior of cells from individual rabbits was quite consistent.

Table 1. Motility of Rabbit Sperm Cells in Minimal Medium

		Incubation Time		
Rabbit	Medium	1-2 (hr)	5-6 (hr)	>20 (hr)
510	S	≤1%,2*	≤1%,2	≤1%,1
	S+BSA	≥40%,4	≥40%,3	≤5%,2
	S+PVA	≥15%,3	≤20%,2	≤10%,2
	S+PVP	≤10%,3	≤5,3	0%
	P	≤15,2	≤25%,3	≤1%,1
	P+BSA	≤50%,4	≤35%,3	≤15%,2
	P+PVA	≤40%,3	≤35%,3	≤10%,2
	P+PVA	≤40%,3	≤35%,3	≤5%,2
	M	≤35%,3	≤25%,3	≤1%,2
	M+BSA	≤55%,4	≤40%,3	≤10%,2
	M+PVA	≤50%,4	≤40%,3	≤15%,2
	M+PVP	≤50%,4	≤30%,3	<1%,1
263	S	0%	0%	0%
	S+BSA	≤30%,3	≤1%,2	0%
	S+PVA	≤10%,1	0%	0%
	S+PVP	0%	0%	0%
	M	0%	0%	0%
	M+BSA	≥70%,5	≤50%,4	≤40%,3
	M+PVA	≤50%,3	≤40%,3	≤15%,3
	M+PVP	≤50%,3	≤40%,3	0%

*Quality of movement on a scale of 1 (poor) to 5 (excellent). Sperm cells were incubated in saline (S), minimal medium (P), or minimal medium buffered with Tris-HCl, pH 7.4 (M) at 37 °C in air, either alone or with BSA (3 mg/mL), polyvinylalcohol (PVA) 1 mg/mL, or polyvinylpyrrolidone (PVP) 1 mg/mL.

3.3 Motility Characteristics.

The changes in motility parameters of sperm cells during a 6 hr incubation in Medium M (Table 2), when hyperactivated motility had ceased, were similar to the changes observed in Medium DM.[7,8] Curvilinear velocity (Vc) was highest at 1 hr but then decreased while there was a continuous decrease

in the average amplitude of lateral head displacement (AALH) with time. The increase in linearity (Lin) at 2 hr is consistent with the cessation of hyperactivated motility at that time. As observed previously,[7,8] the head beat cross frequency (BCF) did not change in a consistent manner over the 6 hr period. Sperm cells hyperactivated in Medium M displayed the four types of hyperactivated motion previously observed in sperm cells hyperactivated in Medium T6.[7] However, in this medium, Type I motion patterns in which the compact 360° trajectory resembled a star or circle were predominant (see Figure). Large numbers of Type IV, biphasic, motion patterns were present, fewer Type III and very few Type II (see Figure).

Table 2. Motility Parameters in Medium M*

	Incubation Time (hr)				
	0.5	1	2	4	6
% Motile	78.9±15.2	80.9±9.4	68.4±23.1	68.6±20.6	49.9±18.4
Vc(μm/s)	71.3±34.0	94.3±35.9	96.4±23.1	91.8±44.3	81.5±44.0
Lin	0.76±0.27	0.78±0.25	0.81±0.17	0.85±0.23	0.85±0.22
AALH(μm)	2.19±2.21	2.35±1.90	2.20±0.69	1.88±1.09	1.61±0.83
BCF(Hz)	16.3±3.57	14.3±4.67	14.6±1.89	15.2±3.84	14.4±4.48

*average ± S.D.

Motion of sperm cells in Medium M was videotaped at time intervals and tapes analyzed by the CellSoft system.

4. DISCUSSION AND CONCLUSION

Elimination of the CO_2-carbonate buffer system for the culture of rabbit sperm cells greatly simplifies the use of rabbit sperm as a model system for in vitro toxicity testing. Volatile compounds can now be easily assayed, and an expensive two or three gas phase incubator is not required for cell culture. Replacement of Medium T6 by Medium M also extends the use of the rabbit sperm in vitro system to include the assessment of the toxic effects of heavy metals or compounds that form insoluble carbonates, phosphates, or sulfates. The precocious hyperactivation of rabbit sperm cells in Medium M also adds another dimension to the use of the rabbit sperm in vitro system in toxicology testing. Hyperactivated motility is necessary for fertilization, and it follows that fertilization failure will result from inhibition of hyperactivated motility. Substances that inhibit this are reproductive toxicants. The expeditious

development of hyperactivated motility by rabbit sperm in Medium M means that this system can be conveniently used for the rapid assessment of the potential antifertility effect of chemicals by measuring the decrease in numbers of hyperactivated cells induced by the chemical.

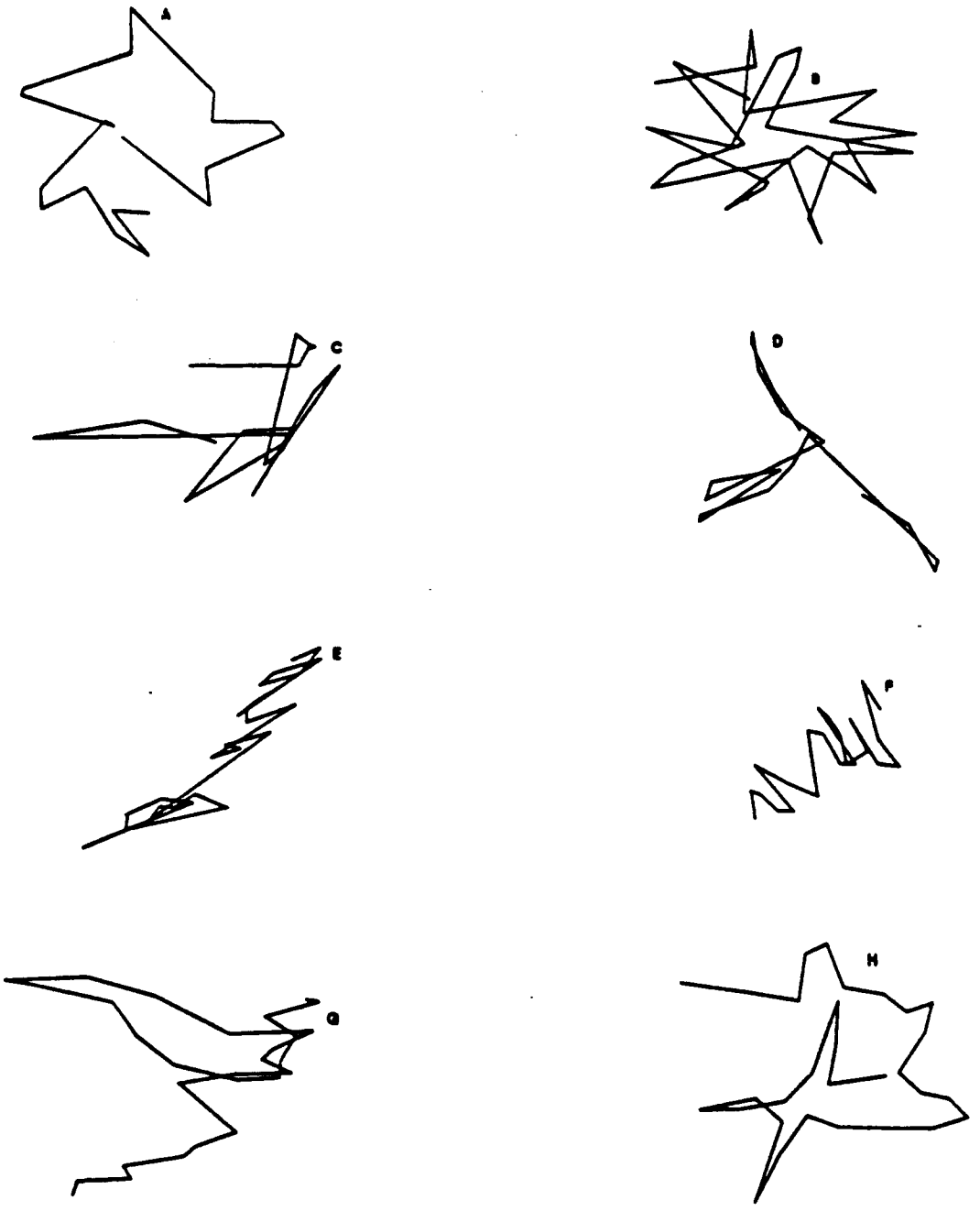

Figure. Trajectory of Hyperactivated Rabbit Sperm. Rabbit sperm cells were incubated in M Medium at 37 °C in air and swimming patterns were recorded on videotape at 30 frames/s after 1 hr incubation. Patterns were analyzed with the CellSoft system.

A = type I, circular; B = type I, star, C, D = Type II; E, F = type II, zig zag; G, H = Type IV, biphasic.

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